

## **Assessing biochar ecotoxicology for soil amendment by root phytotoxicity bioassays**

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# 1    **Ecotoxicological assessment of biochar quality by using phytotoxicity bioassays**

2

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19

## 20    **Abstract**

21    Soil amendment with biochar has been proposed as effective in improving agricultural land fertility  
22    and carbon sequestration, although the characterisation and certification of biochar quality are still  
23    crucial for agronomic acceptance. Described here are the effects of four biochars (conifer and  
24    poplar wood, grape marc, wheat straw) at increasing application rates (0.5, 1, 2, 5, 10, 20, 50%  
25    w/w) on both germination and root elongation of *Cucumis sativus* L., *Lepidium sativum* L. and

26 *Sorghum saccharatum* Moench. Biochars varied in chemical properties, depending on the type and  
27 quality of the initial feedstock batch; PAHs were high in conifer and wheat straw, while Cd and Cu  
28 exceeded maximum permitted values for amendments in poplar and grape marc, respectively. With  
29 our chars, electrical conductivity and Cu altered both germination and root elongation at  $\geq 5\%$  rate,  
30 together with Zn at  $\geq 10\%$  and elevated pH at  $\geq 20\%$ . Germination decreased only at very high rates  
31 of grape marc and wheat straw chars, whereas root length was affected already at 0.5% of conifer  
32 and poplar in cucumber and sorghum, with marked impairment in all chars at  $> 5\%$ . As a general  
33 interpretation, it is proposed here a robust root phytotoxicity logarithmic model in sorghum, based  
34 on biochar Zn content, which explains 66% of variability over the whole dosage range tested. We  
35 conclude that metal contamination is a crucial quality parameter for biochar safety, and that root  
36 elongation represents a stable test for assessing phytotoxicity at recommended amendment rates  
37 ( $< 1\text{--}2\%$ ).

38 **Key-words:** Biochar, Feedstock quality, Germination bioassay, Metal contamination, Root  
39 phytotoxicity

40

## 41 1. Introduction

42 Biochar is a carbon-rich by-product resulting from waste plant material burned with little or  
43 no oxygen at very high temperatures, usually between 300 and 1,000 °C (Jeffery et al., 2011;  
44 Verheijen et al, 2010). In recent years, the importance of biochar for soil amendment has  
45 substantially increased, mainly as a response to increased global carbon emissions and deterioration  
46 of agricultural soil quality (Laird 2008; Lehmann, 2007). Due to its high porosity, specific surface  
47 area and carbon content, biochar can decrease nutrient losses and water leaching, and enhance soil  
48 cation exchange and water-retaining capacities (Chan et al., 2007; Lehman 2007). It can also adsorb  
49 and immobilise persistent organic and inorganic pollutants (Beesley et al., 2010; Hale et al., 2011;  
50 Oleszczuk et al., 2012; Fellet et al. 2014) and pesticides (Cao et al., 2011; Zheng et al., 2010) in

51 sediments and soils, decreasing the potentially noxious effects associated with their  
52 bioaccumulation through the food chain.

53 Despite these benefits, knowledge of soil-biochar interactions is still incomplete. Hazardous effects  
54 may derive from phytotoxic compounds, particularly heavy metals and polycyclic aromatic  
55 hydrocarbons (PAHs). Highly dangerous PAHs originate from degradation of lignin and cellulose  
56 during production (Freddo et al., 2012; Kuśmierz and Oleszczuk, 2014; Oleszczuk et al., 2013) and  
57 are adsorbed onto biochar surfaces (Sharma and Hajaligol, 2003). PAHs are of great environmental  
58 concern, due to their toxic, mutagenic and carcinogenic properties, and their presence may  
59 introduce unacceptable environmental, agronomic and human health risks when biochar is spread  
60 for soil amendment (Kuśmierz and Oleszczuk, 2014). Working parameters during burning of  
61 biomass (temperature, oxygen rate, supply feedstock rate, composition) can affect its chemical and  
62 physical properties (Spokas, 2010). Hence, it is essential to develop rapid and reliable procedures  
63 for biochar screening, to highlight the potentially negative effects on plant growth and human health  
64 before large-scale applications.

65 Within this framework, we compared the effects of four biochars obtained from a standardised  
66 gasification process and various feedstock batches, i.e., conifer and poplar wood, grape marc and  
67 wheat straw, on the germination and root elongation of three plant species routinely used in  
68 bioassay tests, i.e., *Cucumis sativus* L., *Lepidium sativum* L. and *Sorghum saccharatum* (L.)  
69 Moench. We aimed at: i) assessing the quality of different feedstocks, ii) identifying the best  
70 species as indicators of potential biochar toxicity, and iii) finding a relationship between biochar  
71 characteristics and phytotoxicity.

72

## 73 **2. Materials and methods**

74

## 75    **2.1 Biochar production and characterisation**

76

77        Biochars were obtained by gasification in a fixed-bed, down-draft, open-core, compact  
78    gasifier (AGT Company, Italy) at 1,200 °C constant temperature. Four feedstock batches were used:  
79    mixed conifer wood (CO), poplar wood (PO), grape marc (GM) and wheat straw (WS). The effects  
80    of biochars were compared with a reference commercial green-waste composted amendment (CA)  
81    obtained after 6-8-month maturation. pH was measured with a glass electrode on 10 g of pulverised  
82    biochar diluted in 25 mL deionised water, after 1 h shaking and subsequent stabilisation.  
83    Concentrations of trace elements were revealed in ~0.1-0.15 g DW homogenised samples after  
84    microwave-acid digestion (Milestone ETHOS 900, Bergamo, Italy). Samples were analysed by  
85    ICP-OES (SPECTRO CirOS Vision EOP, SPECTRO Analytical Instruments KG, Kleve,  
86    Germany). DTPA extraction was also performed following the Lindsay and Norwell protocol  
87    (1978). PAHs were quantified in 3-g samples treated with 50 mL of toluene (Sigma Aldrich, St.  
88    Louis, MO) for 3 h through Soxhlet equipment; the solvent was then evaporated and weighed. The  
89    residue was recovered with 1-2 mL toluene, adsorbed on a silica gel column and recovered by  
90    washing with 5 mL of toluene, concentrated to 0.1-0.5 mL, spiked with 200 µg of diphenyl (Sigma  
91    Aldrich) as internal standard, and injected (1 µL split mode 1/20 ratio) in a GC-MS analyser  
92    (Agilent Technologies, Inc., Wilmington, DE) equipped with a 30-m capillary column (0.25-mm  
93    i.d., 0.25 mcm f.t) connected with a 5-m silica pre-column (i.d. 0.53 mm). MS data were recorded at  
94    70 eV scan mode (41-440 m/z).

95

## 96    **2.2 Phytotoxicity bioassays**

97

98        Three plant species were used for biochar tests, i.e., *Cucumis sativus* L. (cucumber),  
99    *Lepidium sativum* L. (watercress) and *Sorghum saccharatum* (L.) Moench (sorghum). Seeds from  
100    each species were obtained from plants not previously treated with fungicides. Seed vitality was

101 preliminarily assessed at  $25\pm 1$  °C in deionised water, germination rates being generally >90% for  
102 all species. Seed germination and root elongation tests were performed according to OECD  
103 regulations (1984). The four biochars and CA were pulverised and mixed with a standard soil (SS)  
104 (70% quartz sand, 20% kaolinite, 10% finely-ground *Sphagnum* peat, pH  $8.0\pm 0.2$ ) at 7 increasing  
105 w/w rates: 0.5, 1, 2, 5, 10, 20 and 50%, in comparison with SS alone, as untreated control. Tests  
106 were performed directly on soil matrices because the elutriates could not always reflect the true  
107 toxicity (Visioli et al., 2013; 2014). Four replicates per treatment were arranged by setting 15 g of  
108 substrate in 9-cm diameter disposable Petri dishes, covered with Whatman #1 filter paper and  
109 wetted with 5 mL of deionised water. Ten undamaged plump seeds were placed on the filter and the  
110 dishes were incubated at  $25\pm 2$  °C in the dark for 72 h. Germination rate was evaluated as number of  
111 complete sprouts ( $\geq 1$  mm long) of total number of seeds; shoot (sorghum only) and root lengths  
112 were also measured with a digital gauge.

113

### 114 **2.3 Statistical analysis**

115

116 To ascertain differences between biochars, CA and SS, “many-to-one” multiple comparisons  
117 were performed with Dunnett’s test (Dunnett, 1955) as a follow-up to the one-way ANOVA  
118 procedure. Both endpoints of the phytotoxicity tests (germination and root elongation) were  
119 compared. When ANOVA revealed differences between CA and biochars, a multiple-comparison  
120 Tukey’s HSD test was carried out. A log-logistic model from the Ritz and Streibig ‘drc’ R package  
121 (2005) was applied to fit the dose response of biochars and CA and to estimate the effective  
122 concentrations ( $EC_{10}$ ,  $EC_{30}$  or  $EC_{50}$ ) responsible for reductions of 10, 30 or 50% in root length. In CO and PO  
123 biochars, the log-logistic model failed, and a linear interpolation was applied within the range 0-  
124 0.5% (which accounted for almost all variability). A multiple linear regression (stepwise),  
125 considering additive effects, was also used to verify the effects of different chemicals in biochars on  
126 the reduction in root length. To facilitate interpretation of the large dataset regarding biochar quality

127 and plant response, multivariate statistical analysis was applied at each tested biochar rate, to reduce  
128 the number of variables by PCA (principal component analysis) and to identify common data  
129 distribution patterns by cluster analysis. Before applying these, data were standardised by  
130 subtracting the mean and dividing by standard deviation within each variable.

131 Factorial discriminant analysis (MDA, Multigroup Discriminant Analysis, with Wilks' lambda and  
132 Pillai's trace tests) and PCA were applied to describe phytotoxicity and biochar quality based on  
133 germination rate and root length, and chemical features of biochars (i.e., pH, EC, total PAHs and  
134 metal rates: Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn). Multivariate data normality was preliminarily verified  
135 by the Shapiro test. Multivariate cluster analysis was used to describe the characteristics of  
136 similarity among biochars and plant species. The data clustering algorithm was agglomerative  
137 (bottom-up) with distance optimisation and similarity (Pearson's correlation coefficient) as  
138 proximity method. The squared Euclidean distance and average link (UPGMA, Unweighted Pair  
139 Group Method using Arithmetic Average) were used as cluster distance and linkage method,  
140 respectively. In dendrograms, the maximum level of homogeneity within groups was calculated  
141 with the method of Calinski and Harabasz (1974). All statistical analyses were performed with R  
142 software (2013) and within MS Excel XLSTAT (Addinsoft, Paris, France).

## 143 144 **3. Results**

### 145 146 **3.1 Chemical characteristics of biochars**

147  
148 Chemical characterisation of the four biochars revealed some differences in pH, metals and  
149 PAH contents (Table 1a,b). All biochars were alkaline, pH varying from 8.6 in poplar to 10.4 in  
150 grape marc. Only PO had a pH similar to that of SS and CA; the others were characterised by strong  
151 alkalinity, with pH generally >10 (Table 1a). GM also showed the highest electrical conductivity  
152 (EC >12 mS cm<sup>-1</sup>), ~70× higher than that of PO and CA.

153 Metal concentrations were generally below the maximum admitted threshold recommended  
154 by Italian legislation for amendments (Italian Legislative Decree 217/2006, in application of Reg.  
155 CE n. 2003 13 October 2003). Metals above threshold were Cd in PO (+4%) and, in particular, Cu  
156 in GM (+60%) (Table 1a).

157 Sixteen PAH species were revealed as the main pollutants, according to their potential mutagenic  
158 properties (EPA, 2008). CO had the highest total PAH rate ( $>30 \text{ mg kg}^{-1}$ ), with considerably higher  
159 concentrations of phenanthrene, anthracene and the carcinogenic benzo(b)fluoranthene compared  
160 with the other feedstocks (Table 1b). GM had the lowest value ( $\sim 5 \text{ mg kg}^{-1}$ ), the order being  
161  $\text{CO} > \text{WS} > \text{PO} > \text{GM}$ .

162

### 163 3.2 Effects of biochars on seed germination and root elongation

164

165 Seed germination of plant species was revealed at each tested biochar and CA rate (Fig. 1).  
166 *Cucumis sativus* was not influenced at low rates, germination being significantly reduced with  
167 respect to controls ( $P < 0.001$ ) at only  $>10\%$  of GM and  $>50\%$  of WS. *Lepidium sativum* showed an  
168 increase in germination rate at all tested dilutions of CO and PO whereas, after initial enhancement,  
169 GM again became severely phytotoxic at  $\geq 5\%$  and WS at  $\geq 50\%$  ( $-40\%$  in germination). Similarly,  
170 in *S. saccharatum* CO and PO did not inhibit germination, but was seriously affected above  $10\%$  of  
171 GM and  $50\%$  of WS ( $P < 0.001$ ). GM was the most phytotoxic char, leading to complete inhibition  
172 of germination at  $\geq 10\%$  in watercress and  $\geq 20\%$  in cucumber and sorghum. CA did not negatively  
173 influence germination at any rate.

174 Root growth was severely affected by biochar amendment (Fig. 2). Root length in *C. sativus*  
175 was significantly reduced at all biochar and tested rates ( $P < 0.001$ ). For this species, a marked fall in  
176 root elongation ( $\sim 80\%$ ) was already observed at  $0.5\%$  of CO and PO, followed by a stable response,  
177 whereas phytotoxicity increased progressively with amendment rate in GM and WS. CA also  
178 caused significant root inhibition compared with the untreated control ( $P < 0.001$ ) at any application



179 rate. Matching the complete inhibition of germination at  $\geq 20\%$  of GM, root elongation was also  
 180 impeded. *L. sativum* showed no inhibition in root length in CO and PO biochars, nor in CA, roots  
 181 even being stimulated at all tested rates ( $P < 0.001$ ); conversely, GM and WS, after initial stimulation  
 182 up to 1% and 2% of application, respectively, inhibited the root growth of this species; at 5% GM  
 183 and 10% WS, root lengths drastically fell, with significant differences with respect to controls  
 184 ( $P < 0.001$ ) (Fig. 2). Root and shoot lengths in *S. saccharatum* decreased gradually with increasing  
 185 application rates of CA, GM and WS ( $P < 0.001$ ), whereas marked impairment was immediately  
 186 observed at the lowest amendment rate (0.5%) for CO and PO ( $P < 0.001$ ) (Fig. 2).

187 Biochar application rates causing 10%, 30% and 50% reductions in root length for each species  
 188 were also estimated (Table SI1). Since estimates for PO and CO fell within the small application  
 189 range of 0-0.5%, in which the greatest variations occurred, they also provided the lowest values at  
 190 the three effective concentrations, i.e.,  $\sim 0.06$ -0.09% ( $Ec_{10}$ ), 0.12-0.18% ( $Ec_{30}$ ) and 0.30-0.44%  
 191 ( $Ec_{50}$ ), depending on species. Low  $Ec$  values were also found in GM in combination with sorghum.  
 192 The  $Ec_{50}$  of GM was quite stable across species (1.6-3.7%), but a larger variation was observed for  
 193 WS (7.1-14.7%). Matching the enhancement effect of all biochars within a 2% application rate (Fig.  
 194 2), *L. sativum* generally had a higher  $Ec$  compared with those of the other species (Table SI1).

195 ANOVA detected significant differences in root elongation between CA and biochars as average of  
 196 application rates. Pairwise comparisons confirmed great reductions in length with CO in *C. sativus*  
 197 and with GM in *L. sativum*; generalised growth impairment was observed in *S. saccharatum* (Table  
 198 SI2).

199 Stepwise forward linear multiple regression applied to the whole dataset identified only  
 200 electrical conductivity as a significant char parameter negatively related to root length, but the  
 201 model explained only slight variability ( $R^2 = 0.15$ ). When analysis was broken down by species, the  
 202 Zn biochar rate appeared as the most important variable in both cucumber and sorghum, the  
 203 coefficients of determination being 0.36 and 0.39, respectively. A logarithm model in sorghum  
 204 turned out to provide the most suitable and significant fit ( $R^2 = 0.66$ ) to describe root length (RL, %

205 of unamended control) over Zn concentration ( $\text{mg kg}^{-1}$ ) corrected by biochar rate (BC%), as  
206 follows:

$$207 \quad \text{RL} = 64.162 - 11.81 \cdot \ln(\text{Zn} \cdot \text{BC}\%)$$

208

### 209 **3.3 Principal component analysis and cluster analysis**

210

211 PCA based on chemical characteristics of biochars and standardised seed germination rate  
212 and root length of plant species identified two dummy factors which explained 100% of variability.  
213 The first factor (F1) accounted for almost all variability, i.e., >89% in biochar classification and  
214 >77% in species classification, depending on amendment rate, so that only F1 is shown in Figs. 3  
215 and 4.

216 At low amendment rates (0.5-1%) of biochar classification, F1 was supported (loadings > |0.5|) by  
217 electrical conductivity (EC), Ni and Cr, and seldom by PAH rate (Fig. 3). Although germination  
218 parameters only became significant at  $\geq 5\%$  amendment, germination was initially negatively  
219 correlated with EC and root length with Ni, Cr and PAHs (Fig. 3). Germination was negatively  
220 affected by EC and Cu at  $\geq 5\%$  amendment rate, together with Zn at  $\geq 10\%$  and elevated pH at  
221  $\geq 20\%$ , as highlighted by the opposite direction of their vectors compared with those of germination  
222 rate and root length. In discriminant analysis, ellipsis overlaps and centroid positions generally  
223 highlighted two different groups, GM with high EC and Cu+Zn with maximum phytotoxicity,  
224 distinguished from the other biochars or commercial amendment. Only at 50% rate could a third  
225 group, represented by WS, be clearly plotted separately, with high pH and relatively high EC and  
226 PAH, capable of markedly reducing germination and root length.

227 With regard to species classification, root length significantly supported Factor 1 at all amendment  
228 rates, together with germination rate up to 2% of application. Both sorghum and cucumber were  
229 classified together as more sensitive species than watercress in highlighting phytotoxicity, with  
230 substantial indifference in species choice at 50% biochar rate only (Fig. 4).

231 At all amendment rates, the hierarchical ascendant classification of biochar-species interaction was  
232 a good descriptor of biochar type, regardless of choice of species (Fig. 5). According to the  
233 maximum level of similarity, the first group included poplar as the safest biochar source, with low  
234 EC, pH and Cu contamination, together with the commercial amendment; the second group  
235 included conifer and wheat straw. Grape marc was classified by itself in the third group, with  
236 critical salinity, pH and metal contamination.

237

238

## 239 **4. Discussion**

240

### 241 **4.1 Feedstock and biochar quality**

242

243 Biochar quality largely depends on the chemical and physical characteristics of the initial  
244 batch waste and gasification/pyrolysis parameters. Feedstock quality includes the rate of ash,  
245 together with plant nutrients and heavy metals, lignin, cellulose and hemicellulose rates, all having  
246 a substantial effect on chemical reactions during the semi-anoxic conditions of combustion and thus  
247 potentially leading to accumulation of undesirable and directly toxic compounds. This implies large  
248 variability in biochar composition and its impact on soil and plants, making difficult its  
249 certification.

250 Metal rates, together with EC and pH, are essential chemical characteristics for establishing biochar  
251 safety (Mukherjee and Lal, 2014; Lehmann and Joseph, 2009). In our experiment, biochar pH was  
252 rather variable but generally high, although we did establish its negative influence on plants at very  
253 high application rates (>50%) and in the literature the alkalinisation effect in the open is reported to  
254 be short-lasting (Lucchini et al., 2014). However, alkalinity is also related to high EC, which may  
255 be unsuitable for seed germination and initial root growth within wider ranges of application (5-  
256 50%). The characteristics of GM char, which was very rich in Cu as a result of common treatments

257 against fungal diseases in vineyards under both organic and conventional types of management,  
258 revealed the full effects of this metal. The illegal Cu rate is expected to have a direct phytotoxic  
259 effect on seedlings and to contribute to raising pH and EC. The good affinity of Cu to organic  
260 matter may mitigate its toxicity, but this probably did not occur in the crystalline structure of  
261 biochar, and the high Zn level also contributed towards GM phytotoxicity.

262 It is true that metal mobility is effectively reduced by high pH through stimulation of metal  
263 adsorption and precipitation (Beesley et al., 2011; Zhang et al., 2013), and we found moderate  
264 DTPA-extractable fractions of all metals in biochars, maximum bioavailability being for Cd in WS  
265 (~18% of total), followed by Cu in CO (~10%) and Zn in PO (~6%) (Table 1a). The considerable  
266 effects of heavy metals in germination occur at high biochar rates, and soluble metal fractions play  
267 a more important role in seedling growth than in completion of germination. Germination is  
268 certainly a delicate phase, but root elongation is more sensitive to adverse external conditions, a  
269 result already found in the case of high levels of soluble toxic metals in hydroponics (Vamerali et  
270 al., 2014).

271 Chemical characterisation of the four biochars revealed differences in PAH concentrations,  
272 conifer CO showing the highest rate (Table 1b). In soils, PAHs degrade slowly, and the high  
273 sorption capacity of biochars can extend their degree of environmental hazard over time (Kuśmierz  
274 and Oleszczuk, 2014; Quilliam et al., 2013), although our PCA results never indicated any  
275 important role of PAHs in seed germination and initial root elongation. Total PAHs includes several  
276 toxic compounds with generally low water solubility, and even the more soluble naphthalene and  
277 fluorene were very low or below detection limits. Root PAH concentrations are hardly altered by  
278 various types of biochars (Brennan et al., 2014), and in our case the absence of any direct  
279 root/substrate contact also excludes the possibility of demonstrating the influence of this compound  
280 class on seedling growth. The negative influence of wood-derived chars observed on root  
281 elongation is probably due to high Ni+Cr+Zn and Cd in CO and PO, respectively, some also  
282 exceeding maximum permissible levels for amendments. In markedly metal-contaminated

283 environments, biochar can also enhance root growth through metal immobilisation, indicating that  
284 metal bioavailability is one of the main limiting factors in plant growth (Brennan et al., 2014).

285

286

## 287 **4.2 Species choice as indicator of biochar toxicity**

288

289 The main problem in biochar management of agricultural land is to identify and standardise  
290 the chemical and physical indicators of quality (Lehmann and Joseph, 2009), in order to relate these  
291 characteristics to the potential ecological and toxicological effects on soil-living organisms and crop  
292 productivity (Lehmann et al., 2011).

293 Germination and root elongation are the most common tests in soil bioassays for checking  
294 compound toxicity (Calvelo Pereira et al., 2010; Chigbo and Batty, 2013; Lin and Xing, 2007).

295 Several authors report that these tests are also effective in assessing biochar contamination  
296 (Rogovska et al., 2012; Solaiman et al., 2012). As plant species may substantially differ in their  
297 sensitivity to contaminants, our results show that seed germination was not greatly affected in all  
298 species, whereas root elongation suffered to an extent which depended on choice of species (Figs. 1,  
299 2). Although germination was reduced only at high biochar rate (e.g., 5-10% for GM), an earlier  
300 response may be expected when metal contamination increases greatly, as in biochars derived from  
301 wood treated with Cu-based preservatives, the Cu rate of which may be up to 60 times higher than  
302 the rate we found (Lucchini et al., 2014). Instead, root length is considerably affected already at low  
303 rates, as cell division/elongation in root tips is very sensitive to soil contaminants (Halušková et al.,  
304 2010).

305 Feedstock quality plays an important role in biochar composition and phytotoxicity, but the  
306 contrasting response among plant species to the same biochar was unexpected, e.g., the root length  
307 of *C. sativus* and *S. saccharatum* was suddenly reduced by wood-based PO and CO biochars. Their  
308 low effective concentration values ( $Ec_x$ ) match recent finding on root elongation inhibition by

wood-based biochars (Jeffery et al., 2011). The behaviour of *L. sativum*, which shows root stimulation under wood-based biochars and CA, the latter also deriving from wood residues, and under low rates of GM and WS, is probably due to its higher tolerance to metal contamination. The *Brassicaceae* include several hyperaccumulator species, and cellular metal chelation is an accepted defence strategy (Anjum et al., 2012). This response suggests that bioassays should focus on the more stable behaviour of sorghum roots across the biochar types and CA, as evidenced by our Zn rate-based logarithm model. In *C. sativus*, Zn contamination was also the most suitable variable of this model ( $R^2 = 0.47$ ), followed by pH ( $R^2 = 0.47$ ), whereas the initial lag interval (up to 2-5% biochar rate) and the contrasting effects of chars in *L. sativum* caused the model to fail. For this species, electrical conductivity was the most constraining factor. *C. sativus* is a sensitive and therefore key plant in phytotoxicity bioassays (Wang et al. 2001), although *S. saccharatum* seems a more informative species for biochar investigations. We were surprised to find that *L. sativum* was less sensitive, but this may partly depend on the relatively good quality of our chars and on the type of vegetal matrix, which affects contaminant mobility.

#### 4. Conclusions

At present, there are no clear indicators for the agronomic acceptance of biochar, mainly due to uncertainty regarding its quality and difficulties in predicting interactions with plants and soil biota. Watercress, cucumber and sorghum can all reveal phytotoxicity by reducing their germination rate, albeit only at very high biochar rates. Higher sensitivity, compatible with recommended amendment rates (1-2%), can be easily retrieved from root elongation data. We demonstrate that metal contamination is the most critical constraint for plant growth, and increased metal loads in agricultural land can also exacerbate metal leaching and subsequent groundwater and food chain contamination. Although results on young seedlings must be confirmed in adult plants, these bioassays provide rapid information for char screening. Again, the lack of real long-term studies on

335 biochar effects in cultivated land, identifying contamination in the feedstock stream before  
336 gasification/pyrolysis remains a crucial step before large-scale applications of biochar become  
337 feasible.

338

339

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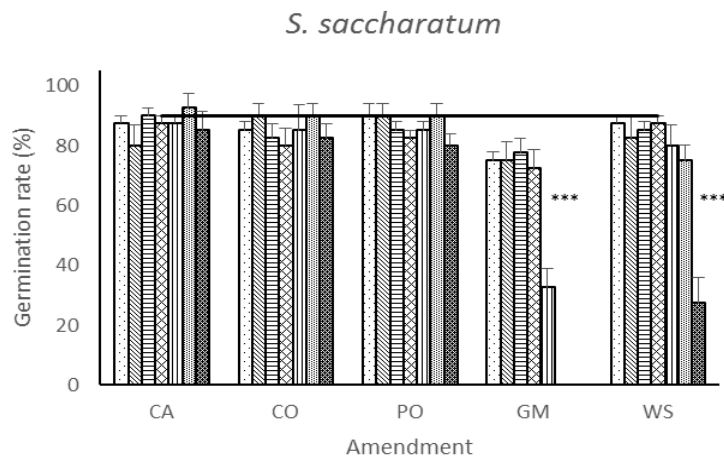
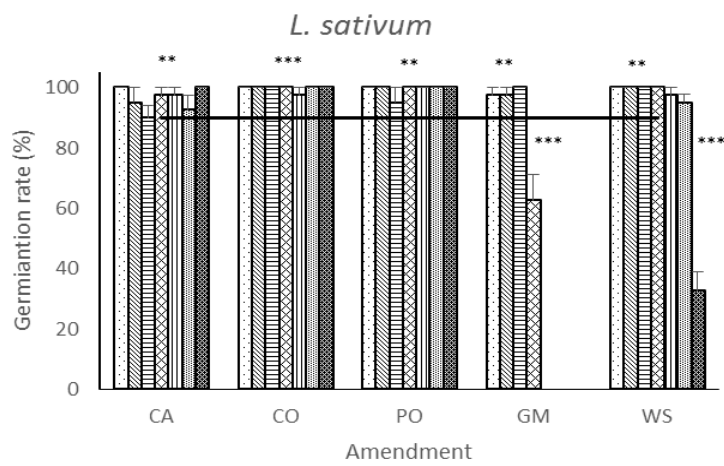
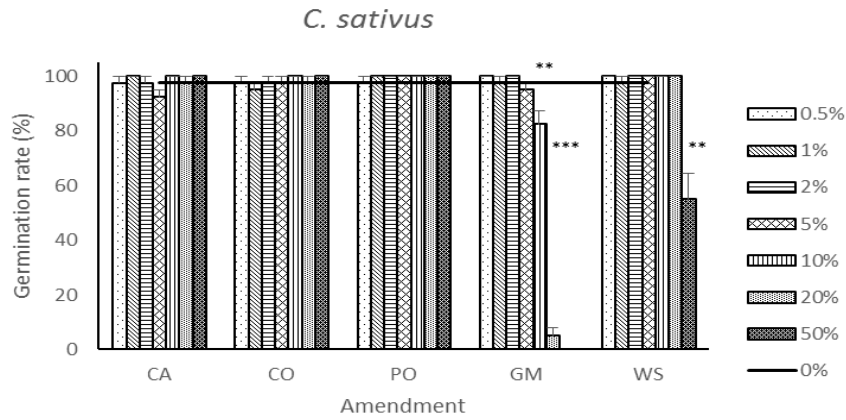
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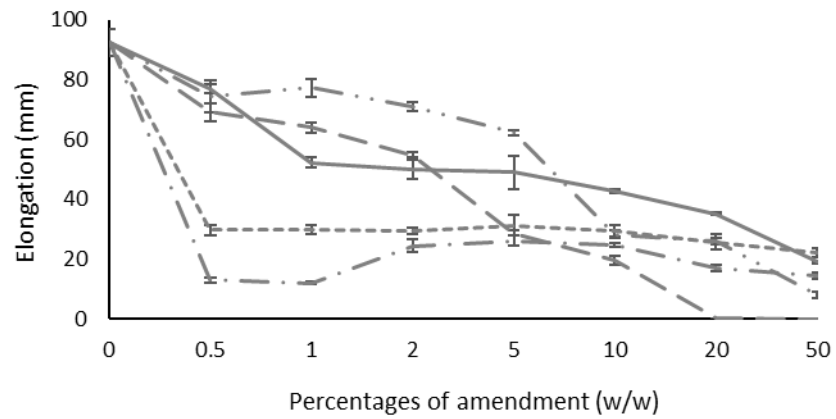
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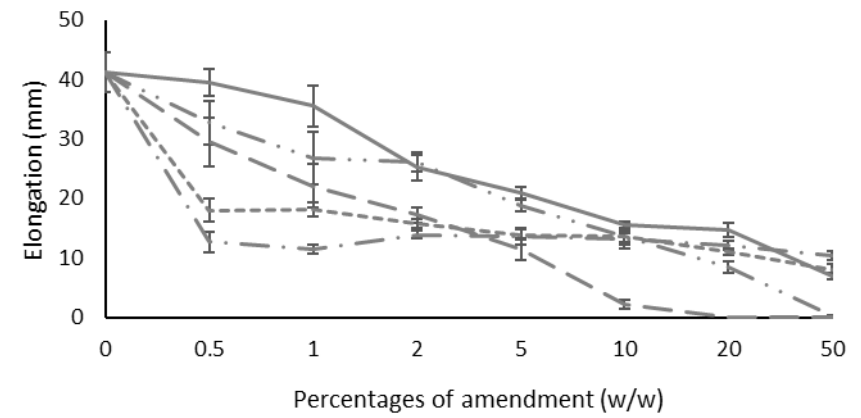
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456 **Figure 1:** Percentage germination (mean value  $\pm$  SE, n = 4) of tested species at different biochar  
 457 rates, compared with commercial amendment (CA) and unamended control (horizontal bar).  
 458 Asterisks: significant differences between amendments and control (\*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

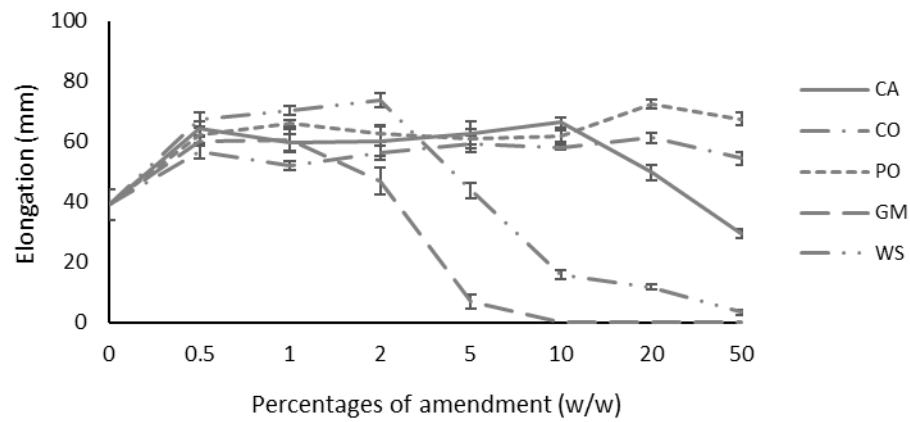
*C. sativus*



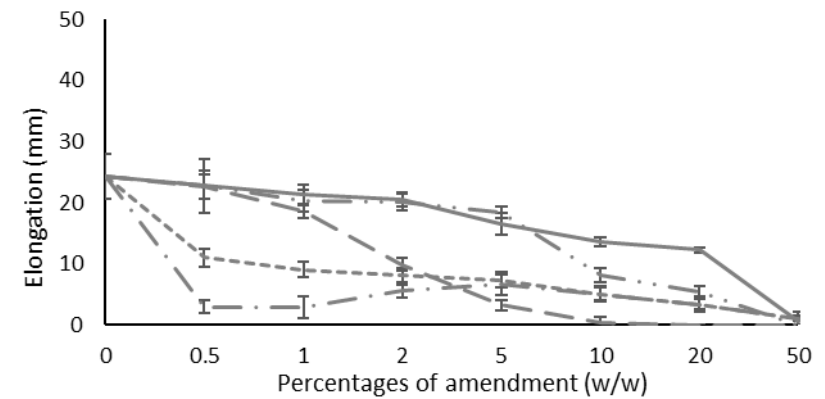
*S. saccharatum* (r)



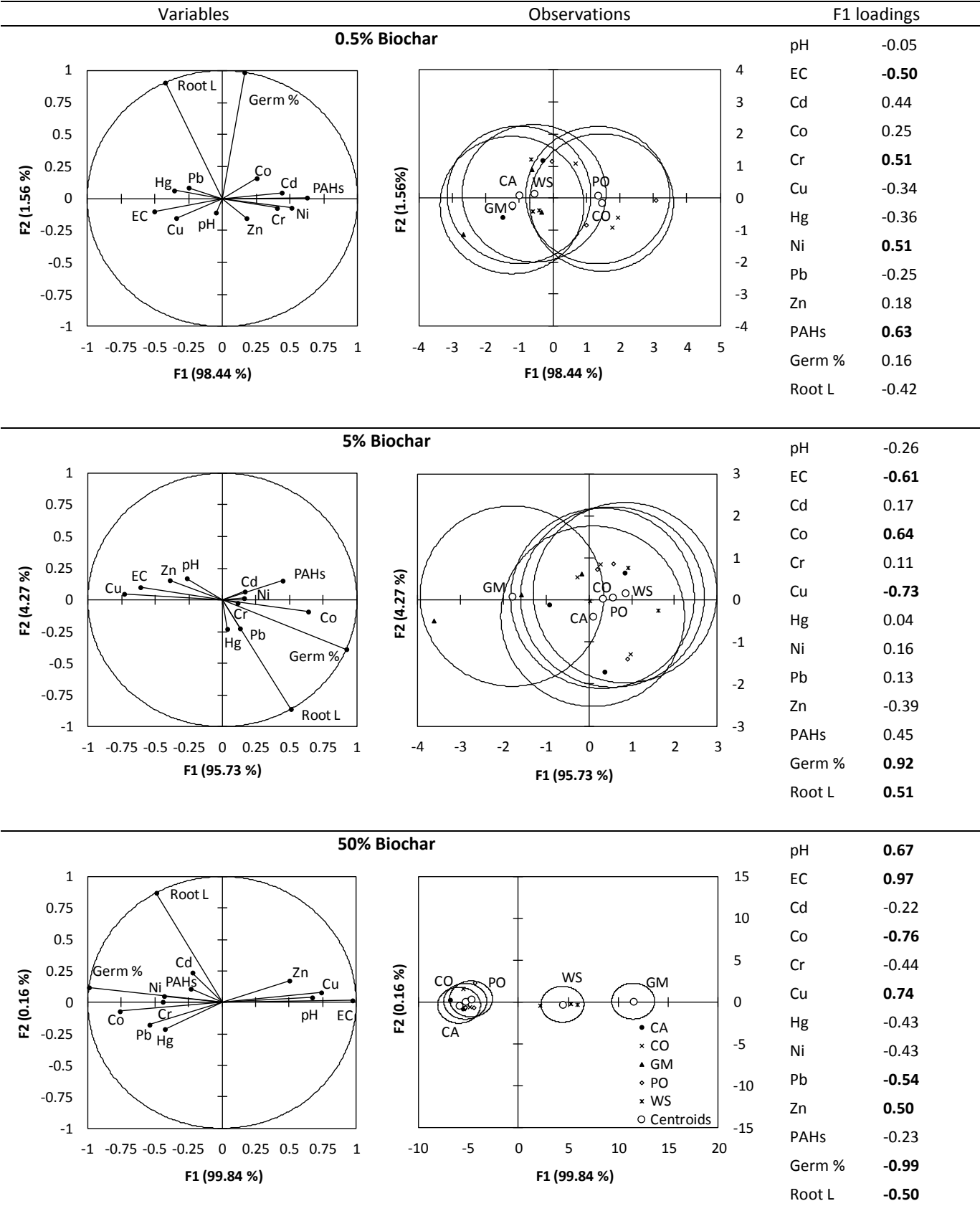
*L. sativum*



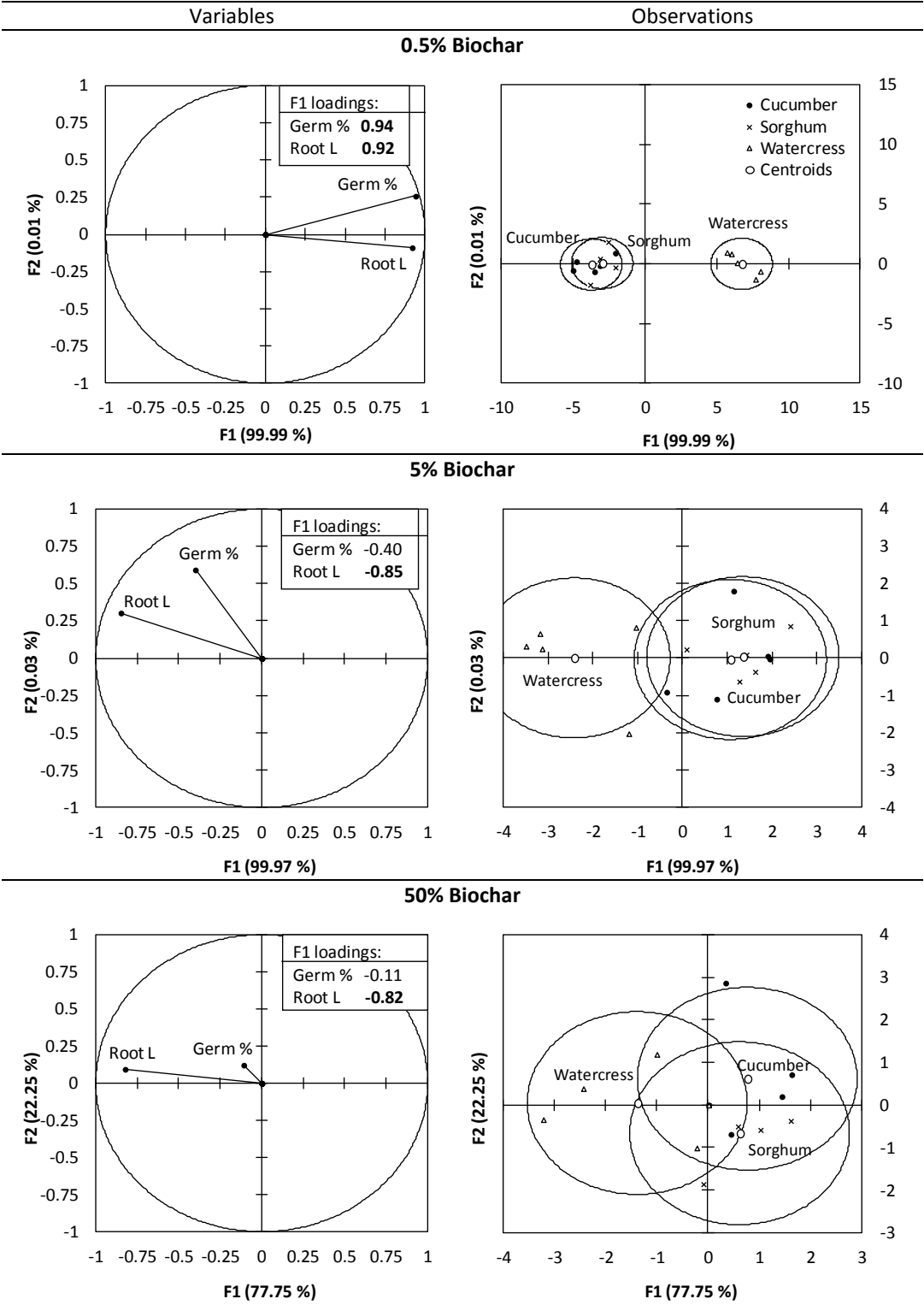
*S. saccharatum* (s)



459 **Figure 2:** Shoot (s) (only sorghum) and root (r) lengths (mean value  $\pm$  SE, n = 4) after germination experiment in three species under increasing  
460 biochar rates, compared with commercial amendment (CA). Note different scale among graphs.

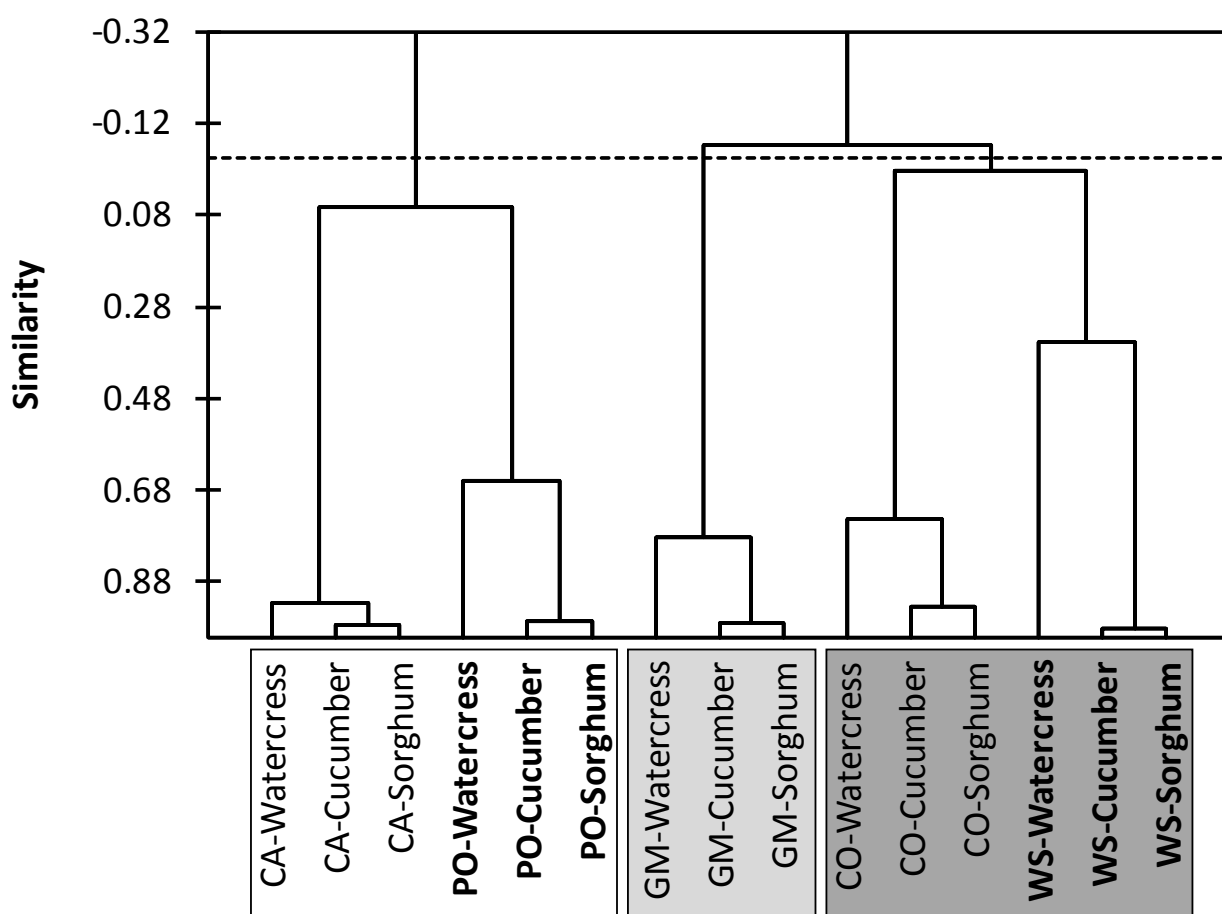


**Figure 3:** PCA with F1 loadings (highlighted values > |0.5|) and DA for biochar classification at 3 selected amendment rates, considering chemical characteristics of biochars, germination rate (Germ %) and root length (Root L).



466 **Figure 4:** PCA and F1 loadings (highlighted values > |0.5|) for species classification at 3 selected  
467 amendment rates, considering germination rate (Germ %) and root length (Root L). Loadings of  
468 chemical characteristics of biochars: always nil.  
469





470

471 **Figure 5:** Cluster analysis of biochar/species combinations at 0.5% amendment rate, based on  
 472 chemical characteristics of biochars and germination rate and root length of 3 species. Horizontal  
 473 dashed line: maximum level of homogeneity within groups. Similar classification obtained at all  
 474 amendment rates.

475

476 **Table 1a:** Main chemical proprieties of biochars in comparison with standard soil (SS) and  
477 commercial amendment (CA)  
478

	IGV	SS	CA	CO	PO	GM	WS
pH	-	8.0	8.2	10.2	8.6	10.4	10.1
EC (mS cm <sup>-1</sup> )	-	ND	0.15	0.62	0.17	12.2	4.79
Cd (mg kg <sup>-1</sup> DW)	1.50	<0.001	0.29	0.34 (12.7)	<b>1.56</b> (14.3)	0.47 (8)	0.41 (17.8)
Co (mg kg <sup>-1</sup> DW)	-	6.13	2.71	2.05 (<3.1)	2.74 (<3.1)	1.29 (<3.1)	2.43 (<3.1)
Cr total (mg kg <sup>-1</sup> DW)	-	89.2	47.1	114 (1)	23.5 (<0.2)	30.3 (<0.2)	29.5 (<0.2)
Cu (mg kg <sup>-1</sup> DW)	230	3.56	53.8	111 (10.1)	34.9 (3.3)	<b>369</b> (1.8)	26.5 (5.5)
Hg (mg kg <sup>-1</sup> DW)	1.50	<0.002	<0.002	<0.001 (-)	<0.001 (-)	<0.001 (-)	<0.001 (-)
Ni (mg kg <sup>-1</sup> DW)	100	53.0	22.0	85.4 (0.7)	18.9 (1.6)	16.2 (0.7)	19.4 (1.8)
Pb (mg kg <sup>-1</sup> DW)	140	7.32	27.7	6.34 (3.6)	10.7 (4.8)	5.23 (<2)	6.92 (2)
Zn (mg kg <sup>-1</sup> DW)	500	25.0	106	272 (4.7)	180 (6.2)	282 (2.2)	183 (4.1)

479  
480 Highlighted values exceed Italian Guidelines Values (IGV) for total metal rate in amendments. In  
481 brackets: % of metal bioavailability (DTPA-extraction).The analysis was performed in duplicate.  
482 ND = not detected  
483

484 **Table 1b:** Polycyclic aromatic hydrocarbons (PAHs) rates (mg kg<sup>-1</sup>) in biochars.  
 485

PAHs	CO	PO	GM	WS
Naphtalene	ND	0.1	0.19	ND
Acenaphthylene	0.27	0.15	2.29	ND
Acenaphthene	0.05	ND	ND	ND
Fluorene	ND	ND	ND	0.19
Phenanthrene	5.29	2.08	ND	1.41
Anthracene	0.93	0.44	ND	0.23
Fluoranthene	3.12	1.28	0.13	4.91
Pyrene	3.80	2.14	0.37	4.95
Benz(a)anthracene	1.40	1.50	0.07	1.22
Chrysene	1.33	0.87	0.09	1.14
Benzo(b)fluoranthene	2.16	1.74	0.06	0.97
Benzo(k)fluoranthene	0.00	1.41	0.10	ND
Benzo(a)pyrene	1.20	1.62	0.10	0.45
Dibenz(a,h)anthracene	ND	ND	ND	ND
Benzo(ghi)perylene	0.88	1.72	0.14	0.27
Indeno(1,2,3-C,D)pyrene	0.63	0.61	0.26	0.09
Total of 16 PAH	21.06	15.66	3.81	15.84
Other PAHs (calculated without response factors)	11.60	5.25	1.24	9.73
TOTAL PAH	32.66	20.91	5.05	25.57

486  
 487 The analysis was performed in duplicate. ND = not detected  
 488

489 **Table SI1:** Effective concentration (%) for biochars at 10, 30 and 50% root length reduction, i.e.,  
490  $Ec_{10}$ ,  $Ec_{30}$ ,  $Ec_{50}$  in three plant species.  
491 n.p. = non phytotoxic  
492

Biochar	Plant species	$Ec_{10}$	$Ec_{30}$	$Ec_{50}$
CO	<i>C. sativus</i>	0.06	0.12	0.30
	<i>L. sativum</i>	n.p.	n.p.	n.p.
	<i>S. saccharatum</i>	0.07	0.14	0.36
PO	<i>C. sativus</i>	0.07	0.15	0.37
	<i>L. sativum</i>	n.p.	n.p.	n.p.
	<i>S. saccharatum</i>	0.09	0.18	0.44
GM	<i>C. sativus</i>	0.3	1.4	3.3
	<i>L. sativum</i>	2.4	3.1	3.7
	<i>S. saccharatum</i>	0.1	0.6	1.6
WS	<i>C. sativus</i>	0.9	3.3	7.1
	<i>L. sativum</i>	3.6	5.0	6.2
	<i>S. saccharatum</i>	0.2	2.8	14.7

493  
494

495 **Table SI 2:** Effects of biochar type on root elongation (main effect, i.e., mean of various  
 496 amendment rates) of plant species at end experiment in comparison with the commercial  
 497 amendment CA (Tuckey's HSD test;  $P \leq 0.05$ ). Letters: statistical significant differences among  
 498 chars within same species.  
 499

Species	Biochar/Amendment	Root length (mm)
<i>C. sativus</i>	CA	46.4 a
	CO	19.1 c
	GM	33.7 b
	PO	33.9 b
	WS	49.7 a
<i>S. saccharatum</i>	CA	19.2 a
	CO	12.5 b
	GM	11.0 b
	PO	13.5 b
	WS	17.8 a
<i>L. sativum</i>	CA	56.1 a
	CO	56.8 a
	GM	24.9 c
	PO	64.9 a
	WS	40.9 b

500